Docket No.: 21058/0206768-US0
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Narayan Sundararajan et al.

Application No.: 10/815,264 Confirmation No.: 7476

Filed: March 31, 2004 Art Unit: 1634

For: MICROFLUIDIC APPARATUS, SYSTEMS, AND METHODS FOR PERFORMING MOLECULAR REACTIONS Examiner: A. Soderquist

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Tae-Woong Koo, declare under penalty of perjury under the laws of the United States of America as follows:

(1) I received a Ph.D. in Mechanical Engineering in 2001 from Massachusetts Institute of Technology. I entered employment as a research scientist for Intel Corporation in 2001 and, in 2005, I was promoted to a manager. My field of research is directed to the use of optical techniques, especially Raman scattering, for the sensitive detection of biomolecules. I am an inventor of the present patent application. I am a co-author of more than a dozen peer-reviewed publications in academic journals, and I have also served as a peer-reviewer for multiple academic journals.

(2) I am familiar with the subject matter and claims of the present application. I am also familiar with the teachings of Dorre et al. (Bioimaging (1997) 5:139-152), Kneipp (US

2002/0150938, Physical Review E 1998, Indian Journal of Physics B), and Ishikawa (Journal of Biophysics 2002) cited by the Examiner in the Office Action of January 23, 2008. Between 1996 and 2001, I worked as a research assistant at George R. Harrison Spectroscopy Laboratory at Massachusetts Institute of Technology, during which time Professor Katrin Kneipp performed single molecule experiments in the same laboratory. I am personally aware of her experiments and the performance of her experimental setup. Professor Kneipp is the first named inventor of Kneipp (US 2002/0150938).

(3) I have reviewed the Examiner's rejection of claims 12-14, 16-17 and 20 over Dorre or Namba in view of Ishikawa or Kneipp in the Action of January 24, 2008. The Examiner rejected claims 12-14, 16-17 and 20 as obvious over the single molecule sequencing apparatus in Dorre or Namba in view of the Raman detection apparatus/method described in Ishikawa or Kneipp. It is my understanding that the Applicants can provide evidence on the record showing that the combination of Dorre or Namba and Ishikawa or Kneipp would be inoperable, that the disclosed invention overcomes the challenge that makes the combination of Dorre and Kneipp inoperable by an unexpected enhancement in detection, and that such evidence may be provided in the form of a Declaration under 37 CFR 1.132. Below, I provide such evidence.

(4) ***Inoperability of the Dorre or Namba apparatus and method when combined with the Raman detector in Kneipp***

Dorre states, "a high signal to noise (S/N) ratio is the essence of single molecule detection (see page 145, col. 1)." The combined Kneipp-Dorre or Kneipp-Namba apparatus/method will ***not*** work and suffer from poor signal-to-noise ratio. Therefore, the combined Kneipp-Dorre or Kneipp-Namba apparatus/method will ***not*** be useful in sequencing ***single nucleotides*** for the following reasons:

(a) The Dorre publication describes an apparatus and the method for sequencing a single nucleic acid molecule with ***fluorescent*** analogs substitute for the normal basis. Dorre provides that

> The fact that single fluorescent molecules can be identified within milliseconds [10] has opened up the horizon for an entirely different approach for sequencing RNA or DNA… This molecule may be degraded sequentially with the help of exonuclease. ***Provided that the released monomers can be detected according to the order of liberation from the polymer substrate, the sequence analysis can be performed at the rate of the enzymatic degradation (≈1-10 bases s-1).*** [Emphasis added.]

Dorre's description emphasizes the emergence of detection techniques that can detect single fluorescent molecules within milliseconds range as a key element to the single DNA/RNA sequencing. ***At the minimum, the Dorre apparatus requires that the detection speed to be equal to or faster than the release speed of the monomers (i.e. nucleotides).*** Otherwise, multiple nucleotides will be detected during a slow measurement and the sequence information is lost. As described by Dorre and as confirmed by Oijen et al. ("Exonuclease Reveal Base Dependence and Dynamic Disorder," Science, 301:1235-1238 (2003)), the speed of exonuclease is not constant. If Dorre's experimental setup releases single nucleotides at the rate between 1 and 10 bases per second, Dorre's experimental setup requires the detection of nucleotides every 0.1 second, which is the inverse of 10 bases per second, or less. For example, when the exonuclease operates to release 10 bases per second, a detector that takes one second will detect the presence of the ten bases without their relative sequence information. As a result, the sequence information will be lost. Therefore, a detection methodology that takes 1 (one) second for each measurement will not be able to detect individual nucleotides coming at the speed between 1 and 10 bases per second, and will not be able to provide the sequence information.

The Kneipp experiment shows in Figure 15 that the detection of single adenine molecule takes one second. Paragraph [0040] of Kneipp '938 provides "FIG. 15 shows typical SERS Stokes spectra representing approximately "1" (top), "0" (middle), or "2" (bottom) adenine molecules in the probed volume where **the collection time is 1 s** and at the excitation radiation is 80 mW near infrared radiation." (emphasis added)." Because the Kneipp method takes one second to detect a single adenine molecule, the Kneipp method cannot be combined with Dorre for the reason discussed above (i.e., the combination will lose the sequence information due to the slow detection). Thus, ***by combining Kneipp's Raman detection apparatus with Dorre's apparatus/method one***

would have simply destroyed Dorre's apparatus/method as the combined apparatus/method would *not* work for Dorre's intended purpose of obtaining sequence information of RNA or DNA by sequentially degrading the RNA or DNA molecule with the help of an exonuclease.

(b) Furthermore, as I am personally aware of the experiments and the experimental setup disclosed in Kneipp, I confirm that the detection speed of the Kneipp method according to Kneipp's apparatus cannot be improved so as to allow detection of ***single nucleotides*** within 0.1 second.

The signal intensity is in general proportional to the measurement time, and the reduction of the measurement time from 1 to 0.1 second will reduce the signal intensity by a factor of 10. However, the noise level in general does not scale in proportion to the measurement time. In low light detections as in the present ultrasensitive Raman detections, the thermal noise and electronic noise inherent in all types of detectors generally determine the level of noise, which does not scale with the measurement time. Therefore, simply running the detector of the apparatus of Kneipp at a faster speed would have reduced the signal intensity proportionally while suffering from the same level of noise, leading to a significantly degraded signal-to-noise ratio to the level that the detection results would not be meaningful. This is consistent with my understanding of the characteristics of Kneipp's experimental setup.

(c) In fact, the detection time of Kneipp's apparatus of 1 (one) second was specifically selected to be 1 (one) second in order to obtain an acceptable signal-to-noise ratio for the detection of a single adenine molecule, which is a base but not a nucleotide. As explained below, Kneipp's experimental setup ***with even 1 (one) second detection time would not be capable to detecting a single nucleotide,*** which is released from a DNA molecule due to the exonuclease activity.

As observed by me as well as other researchers, the intensity of nucleotides, such as deoxyadenosine monophosphate (dAMP), is much weaker than the intensity of bases, such as adenine (See Bell and Sirimuthu, "Surface-Enhanced Raman Spectroscopy (SERS) for Sub-Micromolar Detection of DNA/RNA Mononucleotides," J. Am. Chem. Soc., 125:15580-15581 (2006)). The ability to detect single dAMP molecule versus the ability to detect a single adenine

molecule is critical for sequencing ***single nucleotides***, because the molecules released from a DNA molecule due to the exonuclease activity are nucleotides, such as dAMP, and ***not*** bases like adenine molecules. Kneipp only shows the detection of a single adenine molecule with 1 (one) second detection time, and suggests but does ***not*** show the detection of a single dAMP molecule. Based on the signal-to-noise ratio of the data for detection of adenine molecules as shown in Kneipp's Figure 15, the detection of a single dAMP molecule, which has a weaker signal than that of a single adenine molecule, would have taken significantly longer than 1 (one) second based on Kneipp's apparatus and method.

Because Kneipp's apparatus and method takes at least one second to detect a single adenine molecule and longer to detect a single dAMP molecule, and because the signal-to-noise ratio of Kneipp's apparatus and method in the detection of a single adenine molecule or a single dAMP would be poor if the detection time was within 0.1 second, a person trying to combine Dorre and Kneipp to sequence DNA using a Raman detection would face two unworkable options: increasing the collection time to obtain adequate signal-to-noise ratio in detection of single dAMP molecules at the expense of losing the sequence information, or reducing the detection time with the intent of collecting the sequence information at the expense of significantly degrading the signal-to-noise ratio to an unusable level. Neither of them would have been acceptable for a single molecule sequencer.

For the reasons stated above, the combination of the Raman detection method provided in Kneipp and the single molecule DNA sequencing apparatus based on fluorescence detection as described in Dorre would not work. Given what was known to those of ordinary skill in the art at the time the present application was filed, one of ordinary skill would not have been motivated to, and furthermore could not have combined the Dorre apparatus with the Kneipp apparatus and method.

(d) The Namba apparatus is similar to the Dorre apparatus, and the Kneipp-Namba apparatus will not be operable for the reasons discussed above.

(5) ***Inoperability of the Dorre or Namba apparatus and method when combined with the Raman detector in Ishikawa***

The Ishikawa method is similar to the Kneipp method to the extent that it provides a Raman detector that can detect a single adenine molecule, wherein the "[d]ata accumulation time was 1 s" as explain the description of Figure 7 of Ishikawa. Therefore, the Ishikawa-Dorre or Ishikawa-Namba apparatus/method will not be operable for the reasons discussed above.

For the reasons stated above, the combination of the Raman detection method provided in Ishikawa and the single molecule DNA sequencing apparatus based on fluorescence detection as described in Dorre or Namba would not work. Given what was known to those of ordinary skill in the art at the time the present application was filed, one of ordinary skill would not have been motivated to, and furthermore could not have combined the Dorre or Namba apparatus with the Ishikawa apparatus and method.

(6) ***Unexpected results of this invention: Overcoming the challenge of slow detection faced by Kneipp and Ishikawa***

In order to enable a rapid detection of single nucleotides such that single nucleotides can be identified within 0.1 second, I focused on improving the intensity of SERS signal of single nucleotides at single molecule concentration. Out of many methods I explored, I observed a surprising level of enhancements when the surface enhanced Raman scattering (SERS) and the coherent anti-Stokes Raman scattering (CARS) was combined.

By combining SERS and CARS, which I call surface-enhanced coherent anti-Stokes Raman scattering (SECARS), I observed strong enhancement of the signal compared to when only SERS or only CARS was performed. I found that the SECARS signal of a single molecule of dGMP (90 pM concentration) was at least two orders stronger than the SERS signal for 3×10^8 molecules of dGMP (9 nM concentration) as shown in Figure 1 (below).

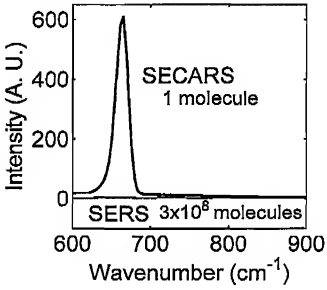

Figure 1. Comparison of SECARS signal from a single molecule and SERS signal of 300 million molecules. CARS, when used alone, could not detect even 300 million molecules.

The SECARS experiment was performed by utilizing the SERS detection simultaneously with the CARS detection. In order to provide the CARS detection capability, a CARS setup is configured in collinear excitation/collection geometry, utilizing two titanium:sapphire lasers, each generating 3 picosecond pulses at 76 MHz repetition rate, synchronized by a phase-locking device (SynchroLock AP, Coherent, Santa Clara, CA). The two lasers were configured to produce the laser pulses of different wavelengths. The laser generating the laser pulse of a longer wavelength is called the Stokes laser. The polarization of the Stokes laser is modulated by a half-waveplate. Two laser pulses are overlapped by a dichroic mirror. The overlapped beam is focused onto a sample by a microscope objective. The back-scattered SECARS signal is collected by the same objective, and both laser lines are blocked by a bandpass filter. The filtered light is sent to a spectrometer, where the dispersion of light is recorded by a liquid nitrogen cooled charge-coupled-device (CCD) camera. The temporal overlap of laser pulses is monitored by the autocorrelator. The tight focusing of the microscope objective relaxes the phase matching condition, and allows SECARS measurements without adjusting the incident angle of the excitation beams for probing multiple vibrational bands.

For each measurement, the Stokes laser was tuned to match the wavelength of a specific Stokes Raman band of the target molecule. Both lasers were operating in pulsed mode, generating picosecond pulses. The lasers were synchronized by a phase-locking device and pulses were temporally overlapped upon reaching the sample. The polarization of the Stokes laser beam was adjusted by tuning the half-waveplate and a polarizer was placed in front of the spectrograph for polarization sensitive SECARS measurements. Prior to the measurements, the half-waveplate and the analyzer were tuned and minimal background was attained.

Colloidal silver suspension was prepared by citrate reduction as known in the field. For each measurement, a solution of target chemical species was first mixed with the silver colloids. A lithium chloride salt solution was then fully mixed into the silver colloid-target molecule mixture, and SECARS spectra were immediately collected.

The strong electromagnetic enhancement of SERS combined with the coherent scattering process of SECARS allows detection of dGMP at single-molecule levels. The data represented in Figure 1 corresponds to from about 10^8, or eight orders of magnitude, to about 10^{10}, or ten orders of magnitude, enhancement of the SERS signal by employing the SECARS method. With this magnitude of unexpected enhancements, I was able to achieve the detection time of 0.1 second and less.

(6) I declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the instant application or any patent issued thereupon.

April 24, 2008
Date

Tae-Woong Koo